IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Gotwals, et al.

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Filed: October 12, 2000 Examiner: Janet L. Andres Ph.D.

Title: TYPE II TGF-BETA RECEPTOR/IMMUNOGLOBULIN CONSTANT REGION FUSION PROTEINS

Mail Stop Patent Application
Commissioner for Patents
P.O. Box 1450
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DECLARATION OF RICHARD CATE, Ph. D

I, Richard Cate, being duly sworn, depose and state as follows.

1. I am an Applicant named in the above-identified patent application. I have reviewed and am familiar with the contents of the April 20, 2004 Office Action issued in connection with the patent application. I have also reviewed the *Lin* and *Jacobs* references which have been cited by the Examiner in the April 20, 2004 Office Action.

2. In my opinion, as of April 18, 1997, those of ordinary skill in the art would not have been motivated to combine *Lin* and *Jacobs* in the manner suggested by the Examiner. Nor would skilled artisans at the time have had a reasonable expectation that combining *Lin* and *Jacobs* as suggested by the Examiner would yield functional fusion proteins within the scope of my invention.

3. As of April 18, 1997, there were five known mammalian type II receptors of the TGF-β receptor family: TGF-βRII (which binds the ligand TGF-β and is also referred to as TβR-II); ActR-II and ActR-IIB (which bind the ligands activin, inhibin, and some BMPs); BMPR-II (which binds some BMPs); and AMH-RII (which binds AMH (MIS)). *See Josso and di Clemente, Current Opinion in Genetics and Development, Vol. 7, pp. 371-377(June, 1997)*(copy attached).

4. On best present information, besides the TGF-β RII fusion protein produced by the Applicants in connection with the making of the claimed invention, there were no known fusion proteins comprising mammalian type II receptors of the TGF-β receptor family and a constant region of an immunoglobulin, prior to April 18, 1997. As of that date, on best present information, skilled artisans had not combined *Lin* and *Jacobs* in the manner suggested by the Examiner to express fusion proteins within the scope of my invention.

5. Before April 18, 1997, I tried to make Fc fusion proteins comprising the extracellular domains of the rabbit and human AMH-RII receptors and a constant region of an immunoglobulin, but I was unable to express such fusion proteins in eukaryotic cells (i.e. COS cells). In the case of the human AMH-RII receptor, three different DNA constructs encoding human AMH-RII-Fc fusion proteins were made, each using different portions of the AMH-RII extracellular domain, but none were successful in producing the fusion protein. In my opinion, the failure to make the AMH-RII fusion protein reflects the unpredictability associated with expressing any member of the TGF-β receptor family as an Fc fusion protein prior to April 18, 1997.

6. I understand that willful false statements and the like made in connection with this declaration are punishable by fine or imprisonment, or both (18 U.S.C. §1001).

Richard Cate

Richard Cate, Ph. D.

October 13, 2004

With many thanks for your help! Nathalie

June, 1977

Serine/threonine kinase receptors and ligands

Nathalie Josso and Nathalie di Clemente

Serine/threonine receptors transduce signals for the TGF-β family, several members of which, such as decapentaplegic and bone morphogenetic proteins, are involved in early patterning of the embryo. The gene encoding the anti-Müllerian hormone (AMH) receptor has recently been cloned; gene targeting produces the same effects as targeting of the *AMH* gene itself. Another divergent member of the TGF-β family, GDNF, signals through Ret, a tyrosine kinase receptor; binding to Ret requires the cooperation of GDNFR-α. The signal transduction pathway of serine/threonine receptors is now being intensively studied; the immunophilin FKBP-12 and MAD proteins are known to be involved.

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http://biomednet.com/elecref/0959437X00700371

Abbreviations

ALK-1	activin receptor-like kinase-1
AMH	anti-Müllerian hormone
BMP	bone morphogenetic protein
Dpp	decapentaplegic
GDF	growth differentiation factor
GDNF	glial cell line derived neurotrophic factor
GPI	glycosyl-phosphatidyl inositol
MAD	Mothers against Dpp
TGF-β	transforming growth factor-β
Xnr	*Xenopus nodal-related*

Introduction

Protein kinases play an important role in signal transduction by phosphorylating specific amino acids of downstream substrates. Catalytic domains predict what kind of residues will be phosphorylated by a given kinase. Many receptors for hormones and growth factors are membrane-bound tyrosine kinases with reasonably well understood signal transduction pathways. In contrast, until relatively recently, serine/threonine kinase activity had been detected only in cytoplasmic proteins downstream of receptor signalling. Starting with the *Caenorhabditis elegans DAF-1*, a myriad of genes encoding transmembrane serine/threonine kinases have been cloned; almost all act as receptors for members of the TGF-β family. In this review, we discuss some of the recent advances in the field of serine/threonine kinase receptors, focusing primarily on their mode of transduction.

Members of the TGF-β family are found in species ranging from *Drosophila* to human and can be grouped in small clusters progressively diverging from decapentaplegic (Dpp) and its human homolog, bone morphogenetic protein-2 (BMP-2) (Table 1). With the exception of glial cell line derived neurotrophic factor (GDNF; see below), all members of the TGF-β family signal through a receptor complex formed by two distantly related types of serine/threonine kinase proteins. Christened type II and type I on the basis of their molecular weights, two such receptors (TβR-II and TβR-I) have now been functionally characterized [1]. Recombinant type II receptors bind their cognate ligand on their own, whereas type I receptors do so only when co-expressed with an appropriate type II receptor. Dpp/BMP receptors exhibit somewhat different binding properties, insofar that type II receptors bind ligand efficiently only in the presence of type I, which, in turn, can bind free ligand (albeit weakly) in the absence of type II [2].

Table 1

The TGF-β superfamily.

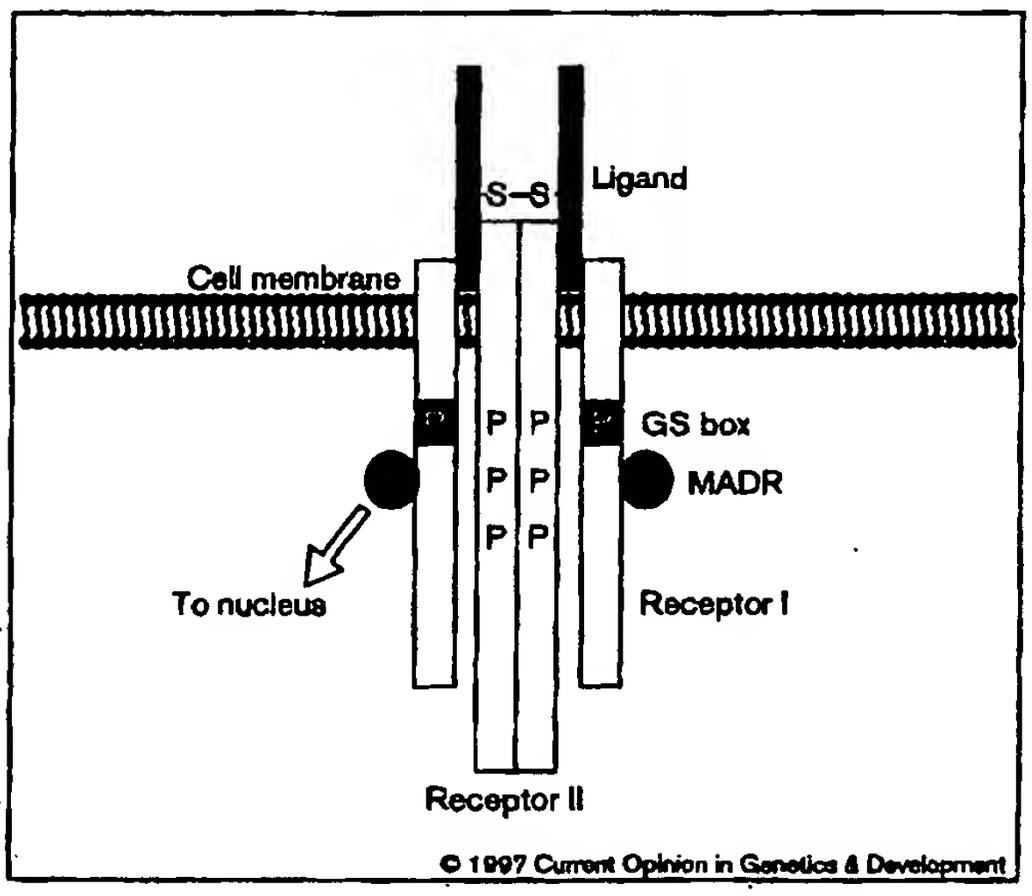
Subfamily	Function
TGF-β subfamily	Growth inhibiton, immunosuppression
TGF-β 1–3	and tissue interactions
TGFβ 4•	
TGFβ 5†	
Activin subfamily	Patterning and regulation of pituitary
Activin β A–C	function
DVR subfamily	Patterning and bone morphogenesis
(includes BMPs)	
Dpp‡, screw‡, BMP2, BMP4	
Vg1†	
60A‡, BMP5, BMP6 (Vgr-1)	
BMP7 (OP-1), BMP8 (OP-2)	
BMP-3 (osteogenin)	
Growth differentiation factors	
GDF-1, GDF-3 (Vgr-2)	
GDF-5 (CDMP-1), GDF-6 (CDMP-2)	
GDF-7, GDF-10 (BMP3-B)	
Dorsalin•	Cell differentiation in the nervous system
Nodal-related subfamily	Induction of mesoderm and right/left
Nodal	patterning
Xnr1–3†	
Divergent members	
AMH/MIS	Sex differentiation
Inhibin	Tumorigenesis and pituitary regulation
GDF-9	Ovarian folliculogenesis
GDNF	Survival of dopaminergic neurons

•Chick; †*Xenopus*; ‡*Drosophila*. DVR, Dpp/Vgl-regulated.

The kinase activity of TβR-II is constitutive and its phosphorylation state is not modified by ligand binding [1]. Ligand binding induces the formation of a receptor complex, most likely a heterotetramer containing two molecules each of TβR-I and TβR-II [3••,4,5•] (Fig. 1). The central event in ligand-induced TGF-β receptor activation is the transphosphorylation of TβR-I by TβR-II at threonines and serines located in the GS box, a

conserved glycine/serine-rich domain located immediately upstream of the kinase consensus domains [6]. Absence of TβR-I, or mutations thereof leading to either loss of kinase activity or inability to be transphosphorylated by TβR-II, blocks signalling responses [3••] (Fig. 2). A missense mutation of TβR-II that blocks the recognition of TβR-I as a substrate has the same effect [7]. In contrast, a mutation within the GS box of several type I receptors yields a constitutively active molecule that signals in the absence of ligand and receptor II [8]. Truncation of TβR-II immediately after the transmembrane domain produces dominant negative molecules which bind to ligand and type I receptors but do not induce biological responses (reviewed in [9•]). The model for activation of the TGF-β receptor complex has recently been extended to activin [10•,11•,12].

Figure 1



Likely mechanisms of signal transduction by serine/threonine kinase receptors. Binding of ligand to receptor type II recruits receptor type I; the complex is probably a heterotetramer containing two molecules of each receptor. The GS box of receptor I undergoes phosphorylation by the receptor II kinase and, in turn, phosphorylates a MAD-related (MADR) protein which then dissociates from the receptor complex and is translocated to the nucleus. Phosphorylation (P) is constitutive on the type II receptor and is induced on the type I receptor and MADR.

Although it contains a transmembrane domain, the type I receptor acts as a substrate and signal transducer for TβR-II, the primary receptor ([13••]; Table 2). The composition of the receptor complex, and more particularly the identity of the type I receptor, determines the nature of the signal. Establishing which type I receptor associates with which ligand/type II receptor complex is tricky because a given ligand may signal through different receptor complexes and a given receptor can recognize different ligands. Furthermore, given that type I and II receptors which probably do not associate under normal cellular conditions may do so when overexpressed in cell lines, binding data alone are not sufficient to assume the existence of a functional signalling complex. Demonstration of co-immunoprecipitation is more reliable as it requires high-affinity association [14]; proof of the capacity of the receptor under investigation to restore signaling in mutant cell lines is even more convincing [15]. The ultimate test, however, is the examination of the phenotypic effects of inactivating the receptor during normal development. In man, mutations have been described only for the anti-Müllerian hormone (AMH) type II receptor [16•] and for the activin receptor-like kinase-1 (ALK-1) [17•]. In the absence of natural mutations, the generation of mutated receptor molecules has proven very useful for dissecting the signalling pathway for TGF-β family members (Fig. 2).

Table 2

Serine/threonine kinase receptors.

Receptors	Main ligand(s)
Type II	
(primary receptors)	
TβR-II	TGF-βs
ActR-II and IIB	Activins, BMP-2, BMP-7, GDF-5
AMH-RII (C14)	AMH/MIS, mutation leads to PMDS
BMPR-II (BRK-3)	BMP-7, BMP-4, GDF-5
Daf-4•	BMP-2, BMP-4
Punt/Atr-II†	Dpp
Type I	
(signal transducers)	
TβR-I (ALK-5)	TGF-βs
ActR-I (ALK-2, Tsk7L)	activin, BMP-7
ActR-IB (ALK4)	Activins
TSR-1 (ALK-1)	?; mutation leads to haemorrhagic telangiectasia
BMPR-IA (ALK-3, BRK-1)	BMP-4, BMP-7
BMPR-IB (ALK-6)	BMP-4, BMP-7, GDF-5
Atr-I†	Activins
sax, tkv†	Dpp
XTrR-I‡	?; overexpression dorsalizes mesoderm
Daf-1•	?; involved in dauer larva formation

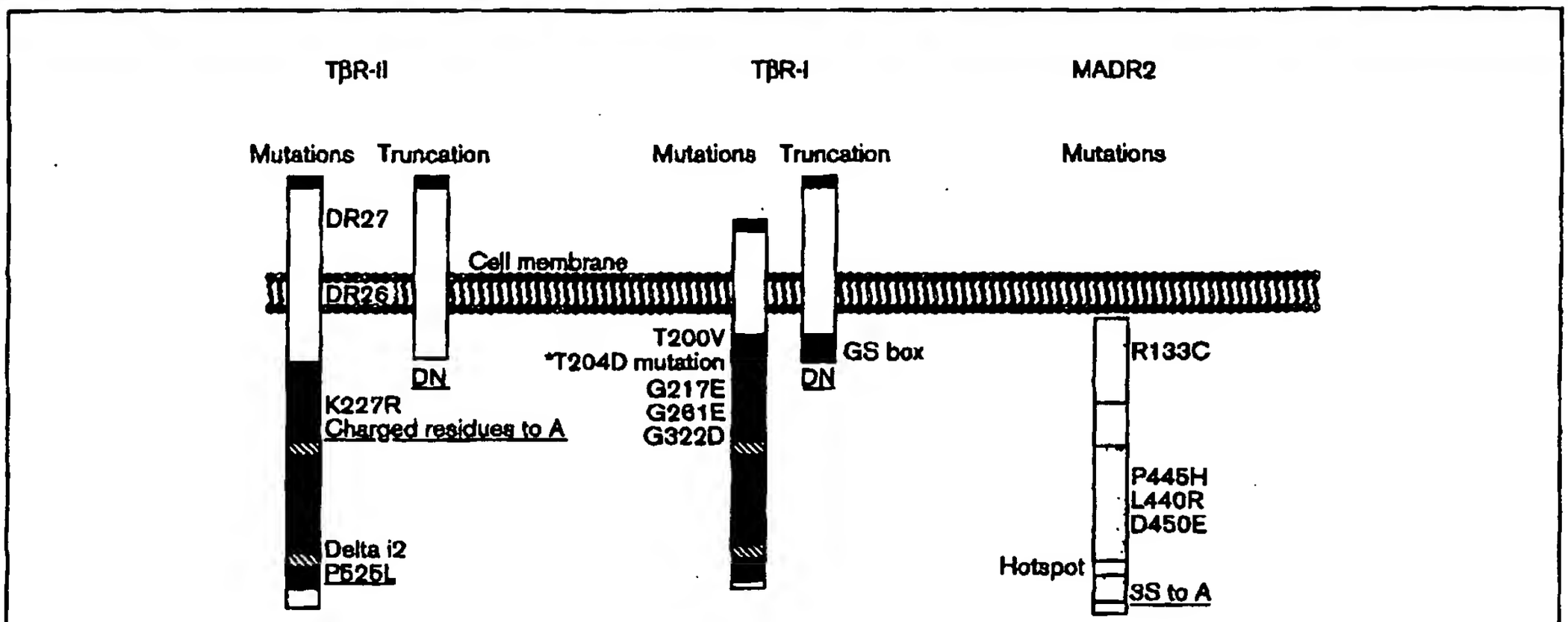
•*C. elegans*; †*Drosophila*; ‡*Xenopus*. ActR, activin receptor; ALK, activin receptor-like kinase; AMHR-II, type II receptor for AMH; Atr, *Drosophila* activin receptor; BMPR, BMP receptor; BRK, BMPR kinase; sax, saxophone; tkv, thick veins; XTrRI, *Xenopus* TGF-β-related receptor type II.

Space limitations do not allow us to discuss all the articles of interest published on the subject of serine/threonine kinase receptors between late 1995 and early 1997. For the same reason, only a handful of older articles have made it to the reference list but an excellent review of 1994 and 1995 literature is available [5•].

Bone morphogenetic factors and their receptors in body patterning

Several members of the TGF-β family are involved in early patterning of the embryo: *Vg1* and members of the *Xenopus nodal-related* (*Xnr*) family [18] can induce dorsal mesoderm formation, the first step towards neuralization. In contrast, BMP4 acts as a mesoderm ventralizer [19]; mutation of cleavage sites yields BMP molecules that are incapable of maturation and leads to dorsalization of the early *Xenopus* embryo [20••].

Figure 2



Mutations that interfere with TGF-β signaling. Mutations that abolish signaling are shown in black and are underlined if dominant negative; a constitutively active mutation is asterisked. TβR-II: K227R [1] is kinase defective; P525L does not allow recognition of TβR-I as a substrate [7]; delta i2, deletion of insert 2 [65]; charged residues to A, mutation of charged residues of the kinase domain to alanine [66]; DN, dominant negative truncated receptor [65,67]. DR26 and DR27 are recessive mutations of the transmembrane and extracellular domains, respectively [68]. TβR-I mutations: DN, dominant negative truncated receptor [66]; G217E (kinase-defective) G261E and G322D (phosphorylation defective) [3]; T204D and T200V [8]. The signal sequence is cross-hatched; the kinase domains are dark shaded; inserts are hatched. MADR2 mutations; conserved amino- and carboxy-terminal domains are light shaded. (Mutations are described in [68••,63••].)

These experiments support the concept that neuralization of embryonic cells, formerly attributed to a positive action of the Spemann organizer, occurs through a default pathway opposed by members of the TGF-β family [20••,21,22•]. Three articles published in the same issue of *Cell* provide evidence that the *Xenopus* embryo derived Spemann organizer products NOGGIN and CHORDIN interrupt DPP and BMP signaling in *Xenopus* [23,24] and also in *Drosophila* [25••] by binding Dpp or BMP-4, thereby preventing them from reaching their respective receptors. In keeping with this hypothesis, NOGGIN does not antagonize the effect of constitutively active forms of the DPP receptors [25••].

These experiments also highlight the conservation of patterning molecules of the TGF-β family between species; despite the large evolutionary distance between insects and vertebrates, both Dpp and BMP4 act in *Xenopus* and *Drosophila* alike [19]. The *Xenopus* factor NOGGIN has biological activity in *Drosophila* and its mechanism of action is the same as in *Xenopus* [25••], although a NOGGIN homologue has not been found in flies. TGF-β family members also influence patterning in fish: a constitutive form of a novel type I receptor is able to induce the most anterior dorsal mesoderm in the zebrafish [26].

During mouse embryogenesis, BMP's and their receptors are essential to mesoderm development [27]. One of the most exciting findings in the field of mouse embryogenesis is that Nodal is expressed asymmetrically in the mouse embryo and may be involved in the establishment of the left–right axis [28••].

Divergent members of the family: anti-Müllerian hormone, inhibin and GDF-9

Three TGF-β family members—inhibin, AMH (also known as Müllerian inhibiting substance/factor) and GDF-9— are preferentially, if not exclusively, expressed in the gonads. All three happen to be relatively remote members of the family, with only 20–30% identity to the family ancestors Dpp/BMP and to TGF-β itself. AMH is responsible for the regression of Müllerian ducts in male mammalian fetuses and also in chick embryos [29,30].

As with all TGF-β family members, AMH requires cleavage at a proteolytic site to exert biological activity. Recent work from the laboratory of Holly Ingraham [31•] suggests that the processing enzyme could be the kex2/subtilisin-like endoprotease PC5. Co-expression of PC5 and AMH in transfected mammalian cells results in efficient processing. PC5 is present in fetal testes, coincident with the beginning of AMH expression. The authors suggest that cleavage of AMH primarily occurs in the testis because, in contrast to previously published work, they did not consistently observe regression in Müllerian ducts exposed to recombinant unprocessed AMH. The concentration in the culture medium, however,

was not accurately determined and could have been too low for detection in the relatively insensitive Müllerian duct bioassay.

As expected for a member of the TGF-β family, AMH probably signals through a pair of related receptors. Although the AMH type I receptor has not yet been identified, the human type II receptor has now been cloned and mapped to chromosome 12. A mutation of a splicing donor site has been detected in a male patient with retained Müllerian derivatives, uterus and fallopian tubes [16•]; failure of the products of the mutant gene to reach the cell surface provides genetic proof that the cloned receptor is functionally involved in AMH signaling [32•]. The phenotype of mutations in AMH or AMH receptor genes is quite similar [33], indicating that no other molecules exert their effects through the AMH receptor and conversely, that AMH does not bind to other receptor complexes. Similar conclusions have been reached independently by the Behringer group [34••], who created a transgenic mouse line in which the AMH receptor has been inactivated by mutagenesis. The phenotype of AMH ligand/AMH double mutant was indistinguishable from those of each single mutant.

Gene knockout experiments have revealed that AMH synergizes with inhibin to influence gonadal tumor development. Inactivation of the AMH [35] or the genes encoding AMH receptor [34••] lead to Leydig cell hyperplasia. Male and female mice in which the inhibin genes have been inactivated by gene targeting develop granulosa/Sertoli cell tumors, first detectable ~4 weeks of age, followed by a wasting syndrome caused by activin signaling [36]. Mice deficient in both inhibins and either AMH [37] or the AMH receptor [34••] developed testicular tumors in both the intratubular and interstitial compartments earlier. Inhibin-deficient mice with concurrent inactivation of gonadotropin-releasing hormone do not develop tumors at all [38].

GDF-9 is expressed only in the oocyte, from the primary follicle to ovulation. GDF-9-deficient female mice are unable to form follicles in spite of the fact that oocyte growth and zona pellucida formation proceed normally [39••]. GDF-9 is the first oocyte-derived growth factor shown to be required for somatic cell function *in vivo*.

GDNF does not signal through a serine/threonine receptor

Another divergent member of the TGF-β family, GDNF, supports the survival of dopaminergic neurons from the midbrain, it is expressed in the nervous system, the gastrointestinal tract and the developing kidney. The identification of GDNF receptor(s) is a consequence of gene knockout technology. Mice with null mutations of the *GDNF* locus [40–42] display renal agenesis and a defect of enteric innervation. There is a striking similarity between this phenotype and that of mice deficient in the protooncogene c-*ret* [43]; furthermore, the gastrointestinal lesions of *GDNF*-null mice mimic Hirschprung's disease which has been traced to loss of function mutations of the human *RET* gene but also to mutations of *GDNF* itself [44].

The similarity between the null phenotypes of *GDNF* and that of the c-*ret* protooncogene led to the suggestion that *ret* could be involved in GDNF signaling. c-*ret* was an unexpected candidate because it is an orphan tyrosine kinase receptor, not likely to be involved in signalling for a member of the TGF-β family. Nevertheless, this surprising hypothesis was soon confirmed experimentally [45•,46•]. Direct binding of GDNF to *ret* requires the cooperation of GDNFR-α, an extracellular protein linked to the cell membrane via glycosyl-phosphatidyl inositol (GPI). GDNFR-α was cloned by expression from a retinal cell library [47••] and from an embryonic rat midbrain library [48]. It binds GDNF with high affinity, is co-precipitated, together with GDNF, by anti-*ret* antibody and mediates *ret* autophosphorylation in the presence of GDNF [47••].

The assembly order and exact composition of the receptor complex for GDNF has not yet been defined. A likely scenario implicates an interaction of GDNF with GDNFR-α. The membrane-linked complex then interacts with two molecules of *ret*, which is then autophosphorylated [47••]. GDNFR-α may have evolved as an adaptor molecule that allowed a tyrosine kinase receptor family member to recognize a new class of ligands.

Downstream of receptors of the TGF-β family: immunophilins and MAD-related proteins

The proteins necessary for signal transduction by members of the TGF-β family are still largely unknown. Yeast two-hybrid screens have allowed the identification of various proteins associating with TGF-β receptors: the WD protein TRIP1 [49]; and the subunit of farnesyl-protein transferase [50], known to play a role in RAS activation.

Table 3

MAD-related proteins in various species.

Drosophila	MAD
C. elegans	smad 2-4
Xenopus	XMAD1-2
Human	SMAD1, MADR1, BSP-1, SMAD2, MADR2, JV18-1
Mouse	Madr2

Another protein interacting in a two-hybrid screen with TβR-I and other type I receptors is the immunophilin FKBP-12. FKBP-12 is a target for the immunosuppressant drugs rapamycin and FK506 and functions as a common inhibitor of the TGF-β family type I receptors [51••]. FKBP-12 binds to ligand-free type I receptors, from which it is released upon ligand-induced activation. Blocking

the interaction between type I receptors and FKBP-12 enhanced responses to TGF-β and to AMH. Release of FKBP-12 from the ligand-bound phosphorylated type I receptor appears essential for the activation of the signalling pathway [51••]. The authors suggest that the inhibitory action of FKBP-12 is not mediated by direct binding to the type I receptor but rather by its ability to dock a cytoplasmic inhibitor, perhaps calcineurin.

Last, but not least, Mothers against Dpp (MAD) and proteins related thereto, have stolen the limelight in the signal transduction scene. The *Drosophila* MAD protein is required for any response to Dpp. Null alleles of *MAD* suppress the dominant phenotype of constitutively active mutants of *thick veins*, which encodes a DPP type I receptor, proving that MAD is an essential component of the downstream signalling pathway of Dpp [52••].

Homologs of the *Drosophila* MAD protein have been cloned in *C. elegans* [53], *Xenopus* [54], mouse [55] and human [52••,56••,57,58••,59] (Table 3). Mutations in *MADR2* have been detected in colorectal cancers [58••] (Fig. 2). A more distantly related protein, DCP4, which associates with MADR proteins, is mutated in pancreatic cancers [60] (Table 3). MADR proteins have no species specificity (reviewed in [61••]), but they do appear to be specific for a given member of the TGF-β family, suggesting that they are dedicated to transducing the signals for a specific subclass of TGF-β ligands [62]. For instance, MADR2 is phosphorylated by TGF-β [58••,63••] and activin [55] and not by BMPs; conflicting data have been reported regarding MADR1 [52••,64]. In *Xenopus*, MADR1 specifies a ventral phenotype similar to the one produced by BMP2 whereas MADR2 induces a dorsal phenotype observed in TGF-β and activin signaling. In *C. elegans*, inactivation of the MAD-related genes *sma* yield phenotypes similar to the one produced by the mutation of *DAF-4*, which encodes a BMP receptor [53].

Although MADR proteins contain no known structural motifs, their amino- and carboxy-terminal domains are highly conserved; most mutations involve invariable residues within a short region close to the carboxyl terminus. Ability to be phosphorylated is crucial to their activity [58••]. MADR proteins are initially distributed equally in the nucleus and the cytoplasm and accumulate in the nucleus after treatment by the appropriate ligand [52••,55,56••].

The recent demonstration that MADR2 physically associates with the TGF-β receptor complex, albeit transiently under physiological conditions, suggests that it acts as a substrate of the ligand-activated TGF-β receptor. In a series of elegant experiments, Jeffrey Wrana, Liliana Attisano and co-workers (now in Toronto) show that interaction and phosphorylation of the protein by the TGF-β receptor occurs via the type I receptor kinase and requires activation of TβR-I by TβR-II [63••]. Normally, MADR2 dissociates from the receptor complex after phosphorylation and redistributes in the nucleus. Mutation of the phosphorylation sites of MADR2 leads to stable association with the receptor complex, failure to accumulate in the nucleus, and lack of signaling. Given that MADR proteins can act as transcriptional activators [56••] and that receptor-mediated phosphorylation drives MADR proteins in the nucleus, it is tempting to speculate that MADR proteins mediate signaling by transmitting the TGF-β signal directly from the receptor complex to the nucleus, where it leads to activation of transcription and initiation of TGF-β responses.

Acknowledgements

We are grateful to Richard Behringer and Richard Cate for a critical reading of the manuscript.

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- • of special interest
- •• of outstanding interest

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